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# Isolation of Gastric Vitamin $B_{12}$-binding Proteins Using Affinity Chromatography

## I. PURIFICATION AND PROPERTIES OF HUMAN INTRINSIC FACTOR*

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## SUMMARY

**Human intrinsic factor has been isolated from gastric juice. The protein was purified 853-fold with a yield of 85% utilizing affinity chromatography on vitamin $B_{12}$-Sepharose as the sole purification technique. The final preparation was homogeneous based on polyacrylamide gel electrophoresis and sedimentation equilibrium ultracentrifugation. The isolated protein (20 μg) corrected vitamin $B_{12}$ malabsorption when given to a patient with pernicious anemia in a standard Schilling test.**

**Human intrinsic factor binds 30.1 μg of vitamin $B_{12}$ per mg of protein and contains a single vitamin $B_{12}$-binding site with an association constant for vitamin $B_{12}$ of $1.5 \times 10^{10}$ $M^{-1}$. The molecular weight determined by sedimentation equilibrium ultracentrifugation was 45,200 to 47,700 while that determined by amino acid and carbohydrate analyses was 44,200. The protein contains 15.0% carbohydrate which accounts for the elevated molecular weight values (59,000 to 66,000) obtained using sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration. In the presence of vitamin $B_{12}$, human intrinsic factor aggregates to form dimers and higher molecular weight oligomers. When vitamin $B_{12}$ binds to human intrinsic factor, the spectral maximum for vitamin $B_{12}$ shifts from 361 nm to 362 nm and the absolute absorbance at 361 nm increases by 32%.**

In many animals including man, the stomach synthesizes and secretes a glycoprotein known as intrinsic factor, which binds vitamin $B_{12}$ and facilitates its absorption in the small intestine in far greater amounts than is possible by the diffusion of free vitamin $B_{12}$ at the levels found in a normal diet (1).

The mechanism by which IF[1] enhances intestinal vitamin $B_{12}$ absorption is not well understood but a number of *in vitro* studies have demonstrated that crude preparations containing IF facilitate the binding of vitamin $B_{12}$ to intestinal sacs (2), homogenates (3, 4), and microvillus membrane preparations (5). This binding is dependent on pH and the presence of calcium ion (6). It is not known, however, whether IF itself is bound to the intestinal mucosal membrane or whether the presence of vitamin $B_{12}$ affects IF binding if it does occur. Uncertainty exists about the role of IF during the entry of vitamin $B_{12}$ into the intestinal mucosal cell since it is not known whether IF, or a portion of the IF molecule, enters the cell with vitamin $B_{12}$ or whether vitamin $B_{12}$ is released from IF at the external cell surface. Uncertainty also exists as to whether other gastrointestinal factors in addition to IF are required for facilitated vitamin $B_{12}$ absorption. The possibility that additional factors are required is suggested by the work of Toskes *et al.* (7, 8) who demonstrated isolated vitamin $B_{12}$ malabsorption in some humans and rats with pancreatic insufficiency. Pancreatic extracts and highly purified preparations of trypsin were able to correct the vitamin $B_{12}$ malabsorption, but the mechanism of action was unclear.

Additional studies concerning the mechanism of IF-facilitated vitamin $B_{12}$ absorption have been severely limited by the difficulties encountered in isolating human IF in homogeneous form. Grasbeck *et al.* (9) succeeded in purifying IF from human gastric juice, obtaining slightly more than 10 mg of homogeneous protein from 40 liters of gastric juice collected over a 2-year period from 376 individuals. Numerous column chromatographic steps were required in their purification scheme. Chosy and Schilling (10) have also purified human IF from gastric juice but uncertainty exists concerning the purity of their final preparation. Many other attempts (11) have been made to purify human IF to homogeneity, but none of these has been successful.

A new method of affinity chromatography has been developed recently (12, 13) in which monocarboxylic acid derivatives of vitamin $B_{12}$ are covalently coupled to 3,3'-diaminodipropylamine-substituted Sepharose using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The vitamin $B_{12}$-Sepharose so formed is an effective affinity adsorbent for a number of vitamin $B_{12}$-binding proteins and has been used to isolate a human granulocyte vitamin $B_{12}$-binding protein (14) and human plasma transcobalamin-II (15) in homogeneous form. Previous work (13) has indicated that this technique might be applicable to isolating human IF. This report is concerned with the purification and properties of human IF isolated from gastric juice using affinity chromatography as the sole purification technique.

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[1] The abbreviations used are: IF, intrinsic factor vitamin $B_{12}$-binding protein; pseudo-vitamin $B_{12}$, α-(adenyl)-cobamide cyanide.

## EXPERIMENTAL PROCEDURE

### Materials

Crystalline vitamin $B_{12}$, bovine serum albumin grade V, whale myoglobin, ovalbumin, rabbit muscle phosphorylase A, and *Escherichia coli* β-galactosidase were obtained from Sigma Chemical Company. Guanidine hydrochloride, ultra pure, was obtained from Mann Research Laboratories. [$^{57}$Co]Vitamin $B_{12}$ was obtained from Abbott Laboratories and Amersham-Searle and had a specific activity of between 10 and 200 μCi per μg of vitamin $B_{12}$. Pseudo-vitamin $B_{12}$ was a gift from Dr. Joseph Pfiffner of Wayne State University. Saliva was obtained from a normal volunteer. Other materials were obtained as described previously (13–15).

### Methods

*Assay of Vitamin $B_{12}$*—Solutions containing [$^{57}$Co]vitamin $B_{12}$ were assayed in a Packard γ scintillation counter. Solutions of nonradioactive crystalline vitamin $B_{12}$ dissolved in water were assayed by measuring the absorbance at 361 nm and 550 nm. Molar extinction coefficients of $E_{1\text{ cm}}$ 361 = 27,700 and $E_{1\text{ cm}}$ 550 = 8,680 were used (16). The values for vitamin $B_{12}$ concentration always agreed within 5% and the average value was used. The vitamin $B_{12}$ content of gastric juice was assayed by the isotope dilution technique of Lau *et al.* (17).

*Assay of Pseudo-vitamin $B_{12}$*—Solutions of crystalline pseudo-vitamin $B_{12}$ dissolved in water were assayed by measuring the absorbance at 361 nm. A molar extinction coefficient of $E_{1\text{ cm}}$ 361 = 27,500 (18) was employed.

*Preparation of Vitamin $B_{12}$-Sepharose*—The preparation and isolation of monocarboxylic acid derivatives of vitamin $B_{12}$ and their covalent attachment to 3,3′-diaminodipropylamine-substituted Sepharose using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide·HCl were performed as previously described (13).

*Assay for Vitamin $B_{12}$-binding Ability*—Vitamin $B_{12}$-binding ability was assayed at room temperature by a modification of the charcoal adsorption method of Gottlieb *et al.* (19). Samples to be assayed (0.05 to 0.20 ml) were adjusted to a volume of 2.0 ml using 0.1 M potassium phosphate, pH 7.5, containing 1 mg per ml of bovine serum albumin and allowed to stand for 20 min. One milliliter of [$^{57}$Co]vitamin $B_{12}$ (1 ng per ml) was added, and after 20 min 2.0 ml of bovine serum albumin-coated charcoal were added to adsorb unbound [$^{57}$Co]vitamin $B_{12}$. After standing for an additional 15 min, the mixture was centrifuged at 20,000 × *g* for 10 min. Three milliliters of the supernatant were counted in a Packard γ scintillation counter. Concentrated samples were diluted in 0.1 M potassium phosphate, pH 7.5, containing 1 mg per ml of bovine serum albumin and allowed to stand at 4° for 30 min prior to assay. The presence of bovine serum albumin served to reduce the adsorption of vitamin $B_{12}$-binding protein to glass that occurs at low protein concentrations. The assay was linear from 0.1 to 0.9 ng of vitamin $B_{12}$-binding ability.

*Pseudo-vitamin $B_{12}$-binding Studies*—The interaction of pseudo-vitamin $B_{12}$ with vitamin $B_{12}$-binding proteins was assayed by measuring the ability of pseudo-vitamin $B_{12}$ to block the binding of [$^{57}$Co]vitamin $B_{12}$ at 4° using vitamin $B_{12}$-binding assays. For each protein studied, a series of test tubes was prepared that contained equal concentrations of vitamin $B_{12}$-binding activity (0.4 to 0.9 ng). One-third of the tubes contained 1.5 ng of nonradioactive pseudo-vitamin $B_{12}$ and another third contained 1.5 ng of nonradioactive vitamin $B_{12}$. No addition was made to the remaining third. After standing for 30 min, 1.0 ml of [$^{57}$Co]vitamin $B_{12}$ (1.0 ng) was added to each tube. All tubes were kept at 4° and after time periods ranging from 0.4 min to 22 hours, 2.0 ml of bovine serum albumin-coated charcoal were added followed in 15 min by centrifugation. The amount of [$^{57}$Co]vitamin $B_{12}$ bound was determined by measuring radioactivity in the supernatant. The decrease in [$^{57}$Co]vitamin $B_{12}$ bound after preincubation with pseudo-vitamin $B_{12}$ was compared with the decrease observed after preincubation with nonradioactive vitamin $B_{12}$.

*Collection of Gastric Juice*—Gastric juice was collected on ice by nasogastric suction from patients with symptoms of gastric peracidity who were undergoing gastric analysis for diagnostic purposes. A nasogastric tube was inserted and the distal end was positioned in the gastric antrum under fluoroscopic observation. Patients were asked to expectorate into a basin rather than swallow their saliva. After a 60-min basal sample was obtained, patients were given Histalog (0.7 mg per kg of body weight) intramuscularly and gastric juice was collected for an additional 90 min. The gastric juice used in this study represented approximately 90%, in terms of volume, of the post-Histalog-stimulated samples. Each sample was kept at 4° and the pH was increased to 10.0 with 5.0 N NaOH to reduce pepsin activity. After standing for 10 min the pH was adjusted to 7.0 with 1.0 N HCl. An aliquot was removed for assay of vitamin $B_{12}$-binding and IF activity and the rest of the sample was stored at −20°. No loss in either vitamin $B_{12}$-binding or IF activity was noted after storage at −20° for periods up to 2 months.

*Preparation of Anti-IF Antibody*—Sera from pernicious anemia patients were screened for the presence of anti-IF antibody using the method of Gottlieb *et al.* (19). Twenty milliliters of anti-IF antibody positive serum from a single patient were passed over a 0.9-cm diameter by 1.0-cm height column of vitamin $B_{12}$-Sepharose at 4° at a flow rate of 10 ml per hour and eluted with 0.1 M potassium phosphate, pH 7.5. The first 30 ml of effluent were brought to a volume of 120 ml with 0.1 M potassium phosphate, pH 7.5, and 72 ml of saturated $(NH_4)_2SO_4$ were added. The sample was stirred for 10 min and centrifuged at 20,000 × *g* for 10 min. The pellet was taken up in 48 ml of solution consisting of 5 parts of 0.1 M potassium phosphate, pH 7.5, and 3 parts of saturated $(NH_4)_2SO_4$ and recentrifuged. The final pellet was dissolved in 45 ml of 0.1 M potassium phosphate, pH 7.5, divided into 5-ml aliquots and stored at −20°. Chromatography on vitamin $B_{12}$-Sepharose and $(NH_4)_2SO_4$ fractionation resulted in the removal of greater than 99.9% of the initial vitamin $B_{12}$-binding activity (2.0 ng per ml) from the serum used. The recovery of anti-IF antibody was approximately 60%.

When a selected sample of normal human gastric juice containing 0.8 ng of vitamin $B_{12}$-binding activity was assayed in the presence of 1.25 μl of the final preparation of anti-IF antibody, vitamin $B_{12}$-binding activity decreased to 0.4 ng. When 10.0 μl of antibody were present, vitamin $B_{12}$-binding activity was reduced to less than 0.004 ng. When comparable amounts of transcobalamin II (15), human granulocyte vitamin $B_{12}$-binding protein (14), and human saliva were assayed for vitamin $B_{12}$-binding activity, no inhibition was observed in the presence of 1.25 to 200 μl of anti-IF antibody.

*In Vitro Assay for IF Activity*—Samples were assayed in duplicate for vitamin $B_{12}$-binding ability as described above. Fifty microliters of anti-IF antibody were added to one of the duplicate tubes at the beginning of the 20-min incubation period prior to the addition of [$^{57}$Co]vitamin $B_{12}$. The percentage de-

crease in vitamin $B_{12}$-binding ability observed with anti-IF antibody was taken as the percentage of vitamin $B_{12}$-binding activity attributable to IF. Except where specifically indicated, IF activity was determined with this assay.

*In Vivo Assay for IF Activity*—IF activity was assayed *in vivo* employing Schilling tests (20, 21) with a patient with pernicious anemia of 9 years duration. [$^{57}$Co]Vitamin $B_{12}$ (0.501 μg, 1.0 μCi per μg), and protein when appropriate, were administered orally in 3.0 ml of 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. The patient was fasted for 8 hours before and 3 hours after the ingestion. Nonradioactive vitamin $B_{12}$, 1 mg, was given intramuscularly at the time of ingestion to saturate the plasma vitamin $B_{12}$-binding proteins and promote the urinary excretion of absorbed [$^{57}$Co]vitamin $B_{12}$. The absorption of [$^{57}$Co]vitamin $B_{12}$ was assayed by measuring the radioactivity present in a 24-hour urine sample started at the time of ingestion. Schilling tests were performed at 3-day intervals. Urine collections made from 60 to 72 hours after each Schilling test demonstrated that less than 0.2% of the oral dose of [$^{57}$Co]vitamin $B_{12}$ was being excreted in the urine at this time.

When Schilling tests are performed with 0.5 μg of radioactive vitamin $B_{12}$, normal individuals excrete greater than 15% of the ingested radioactivity in their urine during the first 24 hours. Patients with pernicious anemia excrete less than 7.5% (22).

*Equilibrium Dialysis*—The association constant for human IF and vitamin $B_{12}$ was measured using equilibrium dialysis. One milliliter aliquots of a solution of 0.1 M potassium phosphate, pH 7.5, containing a constant amount of human IF were placed in Union Carbide dialysis tubing and dialyzed in 6.0-ml plastic test tubes against 4.4 ml of 0.1 M potassium phosphate, pH 7.5, containing 0.025 to 4.0 ng of [$^{57}$Co]vitamin $B_{12}$. After dialysis for 68 hours at 4°, 0.5 ml of the solution in the dialysis tubing and 2.0 ml of each dialysate were removed, and the concentration of vitamin $B_{12}$ was determined by measuring the amounts of radioactivity present. The concentration of vitamin $B_{12}$ bound to human IF (human IF-$B_{12}$) was determined by subtracting the concentration of vitamin $B_{12}$ in the dialysate ($B_{12}$) from the total concentration of vitamin $B_{12}$ in the dialysis tubing. The association constant, $K_A$, is defined as $K_A$ = (human IF-$B_{12}$)/($B_{12}$) (human IF) and was calculated by the method of Steck and Wallach (23) by plotting 1/(human IF-$B_{12}$) *versus* 1/($B_{12}$).

*Protein Assay*—Protein was assayed on samples devoid of vitamin $B_{12}$ by the method of Warburg and Christian (24) which employs measurements of $A_{259}$ and $A_{280}$.

*Protein Concentration*—Samples were concentrated using an Amicon ultrafiltrator equipped with a Diaflo UM-10 membrane.

*Polyacrylamide Disc Gel Electrophoresis*—Protein solutions were subjected to disc gel electrophoresis at pH 9.5 using the standard 7% analytical system (25). Protein samples in water were adjusted to contain 0.003 M potassium phosphate, pH 7.5, 0.05 M NaCl, and 10% sucrose in a volume of 0.16 ml and were layered on top of the gels. Electrophoresis was performed at 4°. Gels were stained for protein with Coomassie brilliant blue.

*Sodium Dodecyl Sulfate Gel Electrophoresis*—Protein samples were adjusted to contain 3% sodium dodecyl sulfate, 0.1 M sodium phosphate, pH 7.4, and 1% 2-mercaptoethanol and were immediately heated for 2 min in a boiling water bath. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, staining, and destaining procedures were performed as described by Baenziger *et al.* (26). Apparent molecular weights were determined by measuring the mobilities of the following proteins of known subunit molecular weight: myoglobin, 17,800; ovalbumin, 46,000; bovine serum albumin, 67,000; phosphorylase A, 94,000; and β-galactosidase, 130,000. The straight line that expresses the empirically determined relationship between log molecular weight and mobility was obtained by the method of least mean squares.

*Absorption Spectra*—Absorption spectra were determined at room temperature in a Cary 15 recording spectrophotometer. Cuvettes with a 1-cm light path were used for all absorbance determinations.

*Molecular Weight Determination by Gel Filtration*—The molecular weight of human IF was estimated using a column (2.0 × 95 cm) of Sephadex G-150, fine grade, equilibrated at 4° with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Samples, in a volume of 6.0 ml of equilibrating buffer containing 10 mg of blue dextran, were applied directly to the top of the column. Calibration of the column with proteins of known molecular weight was performed as previously described (14) except that calibration was in terms of log molecular weight *versus* $V_e/V_0$ where $V_e$ is the peak elution volume of a protein and $V_0$ is the peak elution volume of blue dextran.

*Sedimentation Equilibrium*—The molecular weight of human IF was measured by the meniscus depletion equilibrium method (27) using ultraviolet optics. Cells were scanned at 280 nm. When vitamin $B_{12}$ was bound to human IF cells were also scanned at 362 nm. The partial specific volume was estimated from the amino acid and carbohydrate composition determined on the isolated protein (28, 29). Amino sugars and sialic acid were assumed to be present in their *N*-acetyl form for the purpose of calculating partial specific volumes. When experiments were performed in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl, protein samples were dialyzed against this same solution at 4° for at least 24 hours. Less than 3% of bound vitamin $B_{12}$ was removed during the dialysis procedure. Protein samples studied in 6.0 M guanidine-HCl, 0.08 M potassium phosphate, pH 7.5, were dialyzed against this solution for 48 hours at 22°.

*Amino Acid Analysis*—Protein solutions in distilled water were lyophilized and samples (0.3 to 0.5 mg) were hydrolyzed at 108° for 22 hours in 1 ml of 5.85 M HCl in sealed evacuated tubes. Amino acid and amino sugar analyses were performed using a Beckman model 120C amino acid analyzer. The amount of vitamin $B_{12}$ present was determined by assay of radioactivity in the hydrolysates. Cysteine was determined as carboxymethylcysteine. Methionine was determined after performic acid oxidation (30). Tryptophan was estimated by the method of Edelhoch (31). Free sulfhydryl groups were assayed by the method of Ellman (32).

*Carbohydrate Analysis*—Sialic acid was assayed by the thiobarbiturate method of Warren (33) after hydrolysis in 1 N HCl for 1 min at 100°. Neutral and amino sugars were analyzed by gas-liquid chromatography using the method of Reinhold (34) except for the following modifications. (*a*) Arabinitol and mannitol were used as internal standards; (*b*) hydrolysis in 0.5 M methanolic-HCl for 4 hours at 65° was employed for fucose determinations; and (*c*) hydrolysis in 2.0 M trifluoroacetic acid for 2 hours at 120° followed by hydrolysis in 2.0 M methanolic-HCl for 4 hours at 100° was employed for amino sugar and neutral hexose determinations. Amino sugars and sialic acid were assumed to be present in the *N*-acetyl form when the total weight of carbohydrate per mole of bound vitamin $B_{12}$ was calculated.

*Preparation of Carboxymethylated Protein*—Protein samples

(0.3 to 0.6 mg), devoid of vitamin $B_{12}$, in 2.15 ml of 7.3 M guanidine·HCl, 0.008 M potassium phosphate, pH 7.5, 0.086 M Tris, 0.002 M EDTA, and 0.043 M dithiothreitol were adjusted to pH 9.4 by the addition of 0.22 ml of 1.0 N NaOH. Test tubes containing these samples were flushed with $N_2$, capped, and incubated at 40°. After 8 hours 0.4 ml of 1.0 M iodoacetemide containing 7.5 M guanidine HCl, 0.1 M potassium phosphate, pH 7.5, was added. The measured pH was 8.3. After standing for 20 min in the dark, 0.05 ml of 2-mercaptoethanol was added and the samples were dialyzed against 6.0 liters of distilled water for 72 hours with changes at 4, 24, and 48 hours. The extent of carboxymethylation was monitored by amino acid analyses.

*Purification of Human IF*—The starting material consisted of 3300 ml of gastric juice obtained by pooling collections from 11 different people. Individual samples ranged from 150 to 550 ml in volume, from 12 to 69 μg of total vitamin $B_{12}$-binding activity, and from 89 to 100% in terms of percentage of inhibition of vitamin $B_{12}$-binding activity by anti-IF antibody. The 3300 ml of pooled gastric juice contained 291 μg of vitamin $B_{12}$-binding activity, and 96% of this activity was inhibited by anti-IF antibody. Less than 1 μg of endogenous vitamin $B_{12}$ was present. All procedures were performed at 4°.

Three hundred and thirty milliliters of 1.0 M Tris-acetate, pH 9.2, were added to the 3300 ml of pooled gastric juice and the solution was filtered with vacuum suction through Celite in a Buchner funnel containing a coarse sintered glass disc to reduce the viscosity of the sample. The Celite was subsequently washed with 300 ml of 0.1 M Tris-acetate, pH 9.2, and 3750 ml of combined filtrate were obtained. This was immediately subjected to affinity chromatography on a column, 2.0 cm in diameter and 3.5 cm tall, of vitamin $B_{12}$-Sepharose which contained 4.1 mg of covalently bound vitamin $B_{12}$. The column was washed with 100 ml of 0.1 M Tris-acetate, pH 9.2, immediately prior to the sample application. The sample was applied by gravity at a pressure of 150 cm of $H_2O$. The flow rate was 130 ml per hour. After the entire sample had passed on to the column, the column was washed with 100 ml of 0.1 M Tris-acetate, pH 9.2. The first 3850 ml of effluent were collected in their entirety. The column was then eluted with: (*a*) 2000 ml of 0.1 M glycine-NaOH, pH 10.0, containing 0.1 M glucose and 1.0 M NaCl; and (*b*) 212 ml of 0.1 M potassium phosphate, pH 7.5. The third elution solution consisted of 0.1 M potassium phosphate, pH 7.5, containing 7.5 M guanidine HCl. When 10 ml of this solution had passed through the column, flow was stopped. After 1 hour, an additional 24 ml of eluate were collected, pooled with the first 10 ml, and designated as Eluate 3a. Flow was stopped again and after 17 hours, an additional 24 ml of eluate were collected and designated as Eluate 3b. The starting material, initial column effluent, and each column eluate were assayed for vitamin $B_{12}$-binding activity, IF activity, and protein content. The results are presented in Table I.

Eluate 3a was dialyzed against 250 ml of 0.1 M potassium phosphate, pH 7.5, containing 7.5 M guanidine HCl for 48 hours at room temperature. After dialysis, 1044 μg of [$^{57}$Co]vitamin $B_{12}$ were added and the sample was dialyzed at 4° against 7.0 liters of distilled water for 72 hours with changes at 24 and 48 hours. Greater than 99% of unbound vitamin $B_{12}$ is removed under these conditions.

The final preparation of human IF was stored at −20°. All of the experiments presented below were performed with this preparation.

## RESULTS

*Purification of Human IF*—The precautions taken during the collection of gastric juice are important since they limit contamination with saliva and pancreatic and intestinal juice, all three of which contain significant amounts of vitamin $B_{12}$-binding protein that lack IF activity.

Eluate 3a from vitamin $B_{12}$-Sepharose affinity chromatography was dialyzed against 10 volumes of 7.5 M guanidine HCl, 0.1 M potassium phosphate, pH 7.5, for 48 hours prior to the addition of vitamin $B_{12}$ (see Table I). This dialysis resulted in a decrease in the total absorption at 260 nm and 280 nm, and this resulted in a decrease in the amount of total protein assayed since the method used to assay protein depends on these two parameters. No significant change in vitamin $B_{12}$-binding activity was observed. The nature of the apparent low molecular weight material with absorption at 260 nm and 280 nm removed by dialysis is unknown. Vitamin $B_{12}$ assays (17) were performed

TABLE I

*Affinity chromatography of human intrinsic factor*

| Item | Volume | Vitamin $B_{12}$-binding activity | | | Protein | | Flow rate |
|---|---|---|---|---|---|---|---|
| | ml | mg/ml | total mg | % IF | mg/ml | total mg | ml/hour |
| Filtrate of human gastric juice applied to vitamin $B_{12}$-Sepharose | 3,750 | 77.4 | 290,000 | 96.1 | 2.01 | 7,540 | 130 |
| Initial vitamin $B_{12}$-Sepharose effluent | 3,850 | 0.00 | 0 | | 1.84 | 7,080 | |
| Further elutions of vitamin $B_{12}$-Sepharose | | | | | | | |
| 1. 0.1 M glycine-NaOH, pH 10.0, 0.1 M glucose, 1.0 M NaCl | 2,000 | 5.80 | 11,600 | 99.2 | 0.007 | 14 | 200 |
| 2. 0.1 M potassium phosphate, pH 7.5 | 212 | 0.64 | 136 | 100.0 | 0.002 | 0.4 | 100 |
| 3. 0.1 M potassium phosphate, pH 7.5, 7.5 M guanidine HCl | | | | | | | |
| a. Initial eluate | 34.5 | 6,990 | 241,000 | 99.5 | 0.259 | 8.94 | 25 |
| b. Eluted 18 hours after 3a | 24.0 | 40 | 960 | 50.0 | 0.021 | 0.50 | 25 |
| Eluate 3a | | | | | | | |
| 1. After dialysis against 0.1 M potassium phosphate, pH 7.5, 7.5 M guanidine HCl | 27.5 | 8,720 | 240,000 | 98.0 | 0.300 | 8.25 | |
| 2. After the addition of 1044 μg of vitamin $B_{12}$ followed by dialysis against $H_2O$ | 43.8 | 5,670 | 248,000* | 98.0 | 0.190 | 8.25 | |

* Based on vitamin $B_{12}$ content.

TABLE II

*Purification of human intrinsic factor*

| Step | Volume | Vitamin-$B_{12}$ binding activity | | Protein | Specific activity | $A_{280}:A_{361}$ | Fold purified | Yield |
|---|---|---|---|---|---|---|---|---|
| | *ml* | *μg* | *% IF* | *mg* | *μg vitamin $B_{12}$ bound/mg protein* | | | *%* |
| Human gastric juice | 3300 | 291 | 95.9 | 8240 | 0.0353 | | 1 | 100.0 |
| Affinity chromatography on vitamin $B_{12}$-Sepharose | 43.8 | 248[a] | 98.0 | 8.25 | 30.1 | 1.68 | 853 | 85.2 |

[a] Based on vitamin $B_{12}$ content.

TABLE III

*Schilling tests performed with a patient with pernicious anemia*

| [$^{58}$Co]Vitamin $B_{12}$ ingested | Protein ingested | Urine collected for 24 hours following the ingestion of [$^{58}$Co]vitamin $B_{12}$ | | |
|---|---|---|---|---|
| | | Volume | [$^{58}$Co]Vitamin $B_{12}$ content | |
| *ng* | | *ml* | *ng* | *% ingested amount* |
| 501 | None | 1490 | 7 | 1.4 |
| 501 | 8 μg human IF | 1490 | 44 | 8.8 |
| 501 | 20 μg human IF | 1675 | 114 | 22.8 |

on Eluate 3a and revealed values of less than 0.06 μg of vitamin $B_{12}$ per ml. This ruled out the possibility that the low molecular weight material present was vitamin $B_{12}$ that had been hydrolyzed from vitamin $B_{12}$-Sepharose.

Human IF has been purified 853-fold with a yield of 85.2% as summarized in Table II. One milligram of protein binds 30.1 μg of vitamin $B_{12}$, has an $A_{280}$ of 1.44, an $A_{361}$ of 0.86, and a ratio of $A_{280}:A_{361}$ of 1.68. The final preparation of human IF is homogeneous based on polyacrylamide gel electrophoresis and sedimentation equilibrium ultracentrifugation. Studies employing anti-IF antibody indicate that at least 98% of the vitamin $B_{12}$-binding activity present in the final material is attributable to IF.

*Removal of Vitamin $B_{12}$*—Greater than 99% of bound vitamin $B_{12}$ can be removed from human IF by dialysis for 72 hours at 22° against 15 volumes of 0.1 M potassium phosphate, pH 7.5, containing 7.5 M guanidine HCl with dialysate changes at 24 and 48 hours. Human IF devoid of vitamin $B_{12}$ can be stored in this guanidine solution at 4° for at least 2 months without any loss of vitamin $B_{12}$-binding activity. The ability to remove and replace vitamin $B_{12}$ was used to increase the specific activity of [$^{58}$Co]vitamin $B_{12}$ bound to human IF so that studies such as gel filtration could be performed with small quantities of protein.

*Renaturation of Human IF*—The presence of vitamin $B_{12}$ is not required to achieve complete renaturation (*i.e.* restoration of vitamin $B_{12}$-binding ability) of human IF from solutions of 7.5 M guanidine HCl. This is demonstrated in Table I, where Eluate 3a from vitamin $B_{12}$-Sepharose bound 248,000 ng of vitamin $B_{12}$ when a 4-fold excess of vitamin $B_{12}$ was added prior to the removal of guanidine and unbound vitamin $B_{12}$ by dialysis, and essentially the same value (240,000 ng) was obtained when an aliquot was diluted 1:5,000 in 0.1 M potassium phosphate, pH 7.5, and assayed for vitamin $B_{12}$-binding activity using the charcoal adsorption method. The ability to achieve essentially complete renaturation of human IF in the absence of vitamin $B_{12}$ stands in contrast to our observations with human transcobalamin II (15) and the human granulocyte vitamin $B_{12}$ binding protein (14), since for these two proteins, the presence of vitamin $B_{12}$ is required during the removal of guanidine in order to achieve complete restoration of vitamin $B_{12}$-binding ability.

*Schilling Tests*—The results of the Schilling tests are presented in Table III, and they demonstrate that 20 μg of the final preparation of human IF are able to correct vitamin $B_{12}$ malabsorption in a patient with pernicious anemia.

*Interaction with Pseudo-vitamin $B_{12}$*—Samples of human IF, transcobalamin II (15), and human saliva were utilized to study the ability of pseudo-vitamin $B_{12}$ to block vitamin $B_{12}$ binding at 4°. The results of these experiments are presented in Table IV and suggest that human IF has a lower affinity for pseudo-vitamin $B_{12}$ relative to native vitamin $B_{12}$ than does transcobalamin II or the salivary vitamin $B_{12}$-binding protein. This observation is consistent with studies employing gastric juice (36, 37) and suggests that our final preparation of human IF is free of significant contamination with other vitamin $B_{12}$-binding proteins.

*Polyacrylamide Disc Gel Electrophoresis*—In the absence of vitamin $B_{12}$, 25 μg of human IF move on electrophoresis as a single major protein band. Close inspection of the gel (*Gel A*, Fig. 1) revealed that a faster moving minor protein band was also present. The minor band was not observed when the protein sample contained vitamin $B_{12}$ (*Gels B* and *C*, Fig. 1). The nature of the minor protein band, which also was observed in a second preparation of human IF, is unknown, but the fact that it was observed only in the absence of vitamin $B_{12}$ makes it unlikely that it represents a simple contaminant.

When an excess of vitamin $B_{12}$ was added to 25 μg of human IF 5 min before electrophoresis, a single major protein band was observed together with several faint protein bands that were visualized in the upper region of the gel (*Gel B*, Fig. 1). These minor bands were more numerous and prominent (*Gel C*, Fig. 1) when the sample consisted of 25 μg of human IF that had been saturated with vitamin $B_{12}$ for 72 hours prior to electrophoresis. The fact that these bands become close together as one approaches the top of the gel suggests that human IF aggregates to form a series of oligomers in the presence of vitamin $B_{12}$. Apparent oligomer formation of this type was also observed in experiments employing gel filtration and sedimentation equilibrium ultracentrifugation (see below).

*Molecular Weight Determination by Sedimentation Equilibrium*—When human IF devoid of vitamin $B_{12}$ was studied by sedimentation equilibrium ultracentrifugation, a straight line was observed when $\ln A_{280}$ was plotted *versus* $R^2$ (Fig. 2*A*). Using the partial specific volume of 0.721 calculated from the amino acid and carbohydrate analyses (see below) a molecular weight of 47,700 was obtained for human IF under these conditions.

When a sample of human IF saturated with vitamin $B_{12}$ was studied by sedimentation equilibrium ultracentrifugation under

TABLE IV

*Interaction of pseudo-vitamin $B_{12}$ with human vitamin $B_{12}$-binding proteins*

| Item | Nonradioactive compound present during 30-min preincubation | [$^{57}$Co]Vitamin $B_{12}$ bound at different time periods following the addition of 1000 pg | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.4 min | 1.0 min | 2.0 min | 5.0 min | 30 min | 2 hr | 22 hr | |
| | | pg | pg | pg | pg | pg | pg | pg | % |
| Human IF | None | 148 | 160 | 221 | 303 | 550 | 548 | 613 | 100.0 |
| Human IF | 1500 pg pseudo-vitamin $B_{12}$ | 95 | 127 | 168 | 291 | 477 | 537 | 594 | 97.0 |
| Human IF | 1500 pg vitamin $B_{12}$ | 0 | 1 | 1 | 1 | 9 | 16 | 50 | 8.1 |
| Human transcobalamin II | None | 98 | 170 | 221 | 264 | 381 | 441 | 413 | 100.0 |
| Human transcobalamin II | 1500 pg pseudo-vitamin $B_{12}$ | 2 | 5 | 11 | 15 | 35 | 69 | 125 | 30.3 |
| Human transcobalamin II | 1500 pg vitamin $B_{12}$ | 0 | 0 | 1 | 3 | 7 | 17 | 41 | 9.9 |
| Human saliva | None | 102 | 226 | 312 | 417 | 524 | 562 | 539 | 100.0 |
| Human saliva | 1500 pg pseudo-vitamin $B_{12}$ | 0 | 0 | 0 | 0 | 0 | 2 | 7 | 1.3 |
| Human saliva | 1500 pg vitamin $B_{12}$ | 0 | 0 | 0 | 0 | 0 | 1 | 6 | 1.0 |

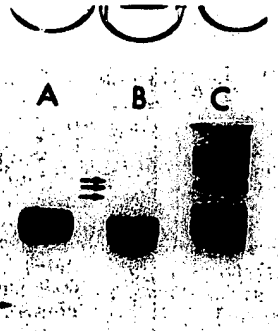


FIG. 1. Polyacrylamide disc gel electrophoresis of human IF in the absence and presence of vitamin $B_{12}$. Protein samples devoid of vitamin $B_{12}$ were renatured from guanidine by dialysis against $H_2O$ for 72 hours at 4°. Protein samples saturated with vitamin $B_{12}$ were renatured as described above, except that excess vitamin $B_{12}$ was added prior to dialysis. *Gel A*, the sample consisted of 25 μg of human IF devoid of vitamin $B_{12}$. The *arrow* indicates the location of a faint protein band that could not be visualized in *Gels B* and *C*. *Gel B*, the sample consisted of 25 μg of human IF and 2 μg of vitamin $B_{12}$. The 2 μg of vitamin $B_{12}$ were added to the protein 5 min prior to electrophoresis. The *arrows* indicate the locations of faint bands that were not visualized in Gel *A*. *Gel C*, the sample consisted of 25 μg of human IF containing 0.82 μg of bound vitamin $B_{12}$. All three gels were subjected to electrophoresis at the same time. At the end of electrophoresis, the tracking dye was just emerging from the bottoms of the three gels.

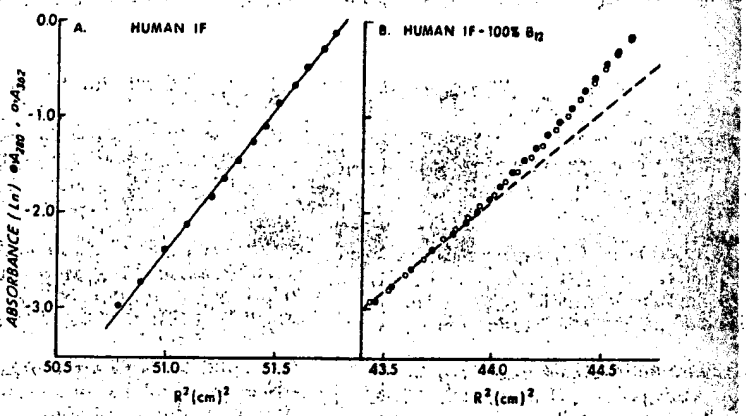


FIG. 2. Sedimentation equilibrium ultracentrifugation studies of human IF in the absence and presence of vitamin $B_{12}$. Experiments were performed in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Protein samples devoid of vitamin $B_{12}$ were renatured from guanidine by dialysis against the salt solution just mentioned for 72 hours at 4°. Protein samples saturated with vitamin $B_{12}$ were renatured in the same way except that excess vitamin $B_{12}$ was added prior to dialysis. *A*, the sample contained 546 μg per ml of human IF devoid of vitamin $B_{12}$. Centrifugation was performed for 24 hours at 5.4° at a rotor speed of 32,000 rpm. *B*, the sample contained 546 μg per ml of human IF and 16.4 μg per ml of vitamin $B_{12}$ (100% saturation of human IF). Centrifugation was performed for 24 hours at 6° at a rotor speed of 30,000 rpm.

similar conditions, plots of ln $A_{280}$ *versus* $R^2$ and ln $A_{362}$ *versus* $R^2$ revealed significant upward curvature (Fig. 2*B*). The plot of ln $A_{280}$ *versus* $R^2$ showed the same degree of curvature as the plot of ln $A_{362}$ *versus* $R^2$, thus indicating correspondence between protein and vitamin $B_{12}$. When portions of the curves shown in Fig. 2*B* were employed for molecular weight calculations, values ranging from 55,000 to 85,000 were obtained. These values indicate that human IF existed as a mixture of monomers and higher molecular weight oligomers under the conditions of this experiment.

A sample of carboxymethylated human IF (150 μg per ml) devoid of vitamin $B_{12}$ was studied by sedimentation equilibrium ultracentrifugation in 6.0 M guanidine HCl containing 0.08 M potassium phosphate, pH 7.5. Sedimentation was performed for 44 hours at 8.8° at 36,000 rpm. The plot of ln $A_{280}$ *versus* $R^2$ was linear. Using the partial specific volume of 0.721 a molecular weight of 45,200 was obtained, indicating that smaller molecular weight subunits do not exist for this protein.

*Amino Acid and Carbohydrate Composition*—The results of the amino acid and carbohydrate analyses are presented in Table V. Using the molecular weights of the individual amino acids and carbohydrates, human IF contains 44,200 g of amino acid and carbohydrate per mole of bound vitamin $B_{12}$. This value is close to the monomeric molecular weight value of 45,200 to 47,700 obtained for human IF by sedimentation equilibrium ultracentrifugation and indicates that the protein contains a single vitamin $B_{12}$-binding site.

TABLE V

*Amino acid and carbohydrate composition of human IF*

Amino acid analysis was performed on duplicate 22-hour hydrolysates and carbohydrate analysis was performed by gas-liquid chromatography as described under "Methods."

| Item | Residues per mole of bound vitamin $B_{12}$ | Item | Residues per mole of bound vitamin $B_{12}$ |
|---|---|---|---|
| Amino acid | | Amino acid | |
| Lysine | 20 | Tyrosine | 9 |
| Histidine | 5 | Phenylalanine | 10 |
| Arginine | 6 | Methionine | 10[a] |
| Aspartic acid | 38 | Half-cystine | 6[b] |
| Threonine | 24 | Tryptophan | 6[c] |
| Serine | 30 | Carbohydrate | |
| Glutamic acid | 35 | Fucose | 7 |
| Proline | 22 | Galactose | 6 |
| Glycine | 20 | Mannose | 12 |
| Alanine | 23 | Galactosamine | 3 (2)[d] |
| Valine | 22 | Glucosamine | 6 (4)[d] |
| Isoleucine | 22 | Sialic acid | 3[e] |
| Leucine | 34 | | |

[a] Determined as methionine sulfone after performic acid oxidation.

[b] Determined as carboxymethylcysteine. Accurate quantitation as cysteic acid was not possible since ninhydrin-positive material was present in the cysteic acid position in the absence of performic acid oxidation.

[c] Determined spectrophotometrically.

[d] Values in parentheses were determined using the amino acid analyzer.

[e] Determined by the thiobarbiturate method.

The sulfhydryl group content of human IF was assayed in 7.5 M guanidine HCl containing 0.1 M potassium phosphate, pH 7.5. No free sulfhydryl groups were detected (<0.1 residue per mole), suggesting that the 6 cysteine residues present in this protein are involved in disulfide bonds.

*Molecular Weight Determination by Gel Filtration*—When 16 μg of human IF devoid of vitamin $B_{12}$ were applied to a calibrated column (2.0 × 95 cm) of Sephadex G-150, a single symmetrical peak of vitamin $B_{12}$-binding activity was observed (Fig. 3*A*) with an apparent molecular weight of 66,000. This value is significantly greater than the molecular weight values of 44,200 and 45,200 to 47,700 obtained, respectively, by amino acid and carbohydrate analyses and sedimentation equilibrium ultracentrifugation. The anomalous value for molecular weight obtained by gel filtration is probably attributable to the fact that human IF contains 15.0% carbohydrate since glycoproteins frequently give falsely elevated values for molecular weight when determined by gel filtration (14, 35, 38).

When 17 μg of human IF containing 0.52 μg of [$^{57}$Co]vitamin $B_{12}$ were applied to the same Sephadex G-150 column, three peaks of radioactivity were observed as shown in Fig. 3*B*. The peaks that eluted with apparent molecular weights of 65,000 and 138,000 appear, respectively, to represent monomers and dimers of human IF. It is uncertain whether the material eluted with an apparent molecular weight of 210,000 represents trimers or tetramers of human IF, since molecular weight values above 200,000 are relatively inaccurate on Sephadex G-150.

When 17 μg of human IF containing 0.52 μg of [$^{57}$Co]vitamin $B_{12}$ in 0.5 ml of Sephadex G-150 buffer were incubated at room temperature for 8 hours prior to application to Sephadex G-150, a pattern of [$^{57}$Co]vitamin $B_{12}$ elution indistinguishable from that shown in Fig. 3*B* was obtained.

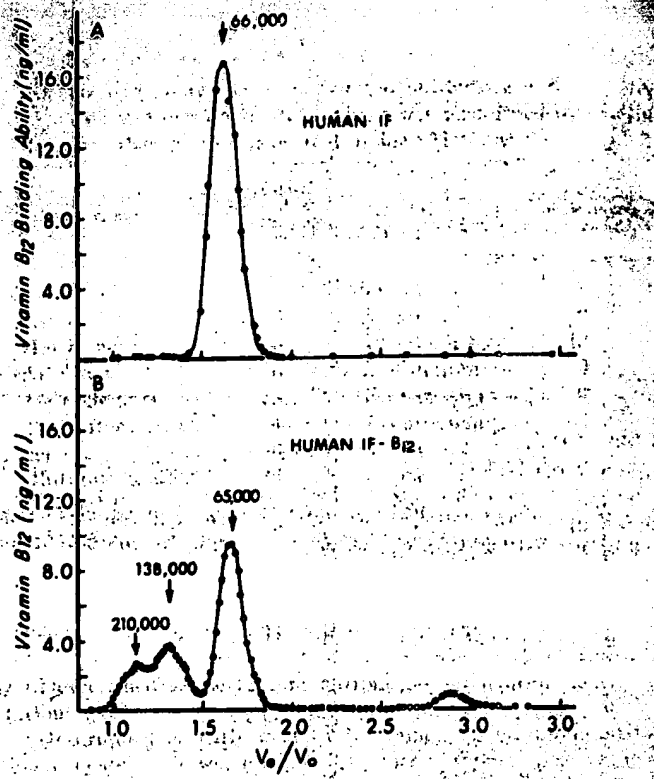


FIG. 3. Gel filtration studies of human IF in the absence and presence of vitamin $B_{12}$. Experiments were performed at 4° using a column (2.0 × 95 cm) of Sephadex G-150 equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl. Protein samples were prepared as described in the legend for Fig. 2. *A*, the sample applied to the column contained 16 μg of human IF devoid of vitamin $B_{12}$. Fractions were assayed for vitamin $B_{12}$-binding activity. The amount of vitamin $B_{12}$-binding activity recovered was 72% of the amount applied. A molecular weight of 66,000 was obtained for human IF based on the elution position of the single observed peak of vitamin $B_{12}$-binding activity. *B*, the sample applied to the column contained 17 μg of human IF and 0.52 μg of [$^{57}$Co]vitamin $B_{12}$. Fractions were assayed for vitamin $B_{12}$ content based on measurements of radioactivity. The amount of radioactivity recovered from the column was 75% of the amount applied. Molecular weights of 65,000, 138,000, and 210,000 were obtained for human IF based on the elution positions of the three observed peaks of vitamin $B_{12}$.

FIG. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of 30 μg of human IF. The *arrow* indicates the direction of electrophoresis.

*Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis*—When 25 μg of human IF were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained for protein, a single band was observed as shown in Fig. 4. The molecular weight estimate based on the position of this band is 59,000 (Fig. 5). This value is similar to the value of 66,000 determined by gel filtration (see above) and suggests that human IF consists of a single polypeptide chain. The value of 59,000 is probably a falsely elevated estimate as has been reported for other glycoproteins studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (14, 35, 39, 40).

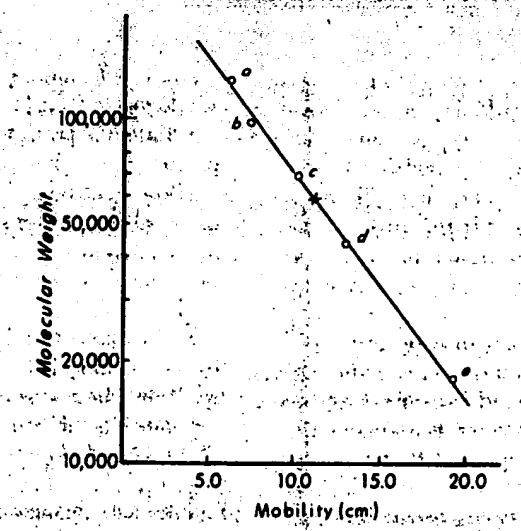


FIG. 5. Determination of the apparent molecular weight of human IF by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins used as standards were: *a*, β-galactosidase; *b*, phosphorylase A; *c*, bovine serum albumin; *d*, ovalbumin; and *e*, myoglobin. × indicates the mobility observed with 30 μg of human IF and indicates an apparent molecular weight of 59,000.

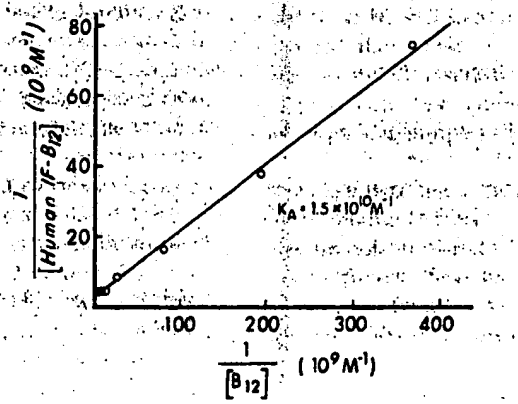


FIG. 6. Determination of the association constant, $K_A$, for human IF and vitamin $B_{12}$ using equilibrium dialysis at 4° in 0.1 M potassium phosphate, pH 7.5.

*Association Constant for Human IF and Vitamin $B_{12}$*—The data obtained from the equilibrium dialysis experiments are presented in Fig. 6. The value of $1.5 \times 10^{10}$ $M^{-1}$ obtained for the association constant, $K_A$, for human IF and vitamin $B_{12}$ is similar to the value of $0.38 \times 10^{10}$ $M^{-1}$ obtained by McGuigan (41) under similar conditions using human gastric juice.

*Absorption Spectra*—The spectrum of the human IF-vitamin $B_{12}$ complex together with the spectra of the same concentration of unbound vitamin $B_{12}$ and the same concentration of human IF devoid of vitamin $B_{12}$ are presented in Fig. 7. When vitamin $B_{12}$ binds to human IF the spectral maximum for vitamin $B_{12}$ shifts from 361 nm to 362 nm.

Fig. 7 also indicates that the absorption of the human IF-vitamin $B_{12}$ complex above 320 nm is generally greater than the sum of the absorption of unbound vitamin $B_{12}$ and human IF devoid of vitamin $B_{12}$ when the absorption of the latter two are measured at the same concentration as that of the human IF-vitamin $B_{12}$ complex. The absorption of the human IF-vitamin $B_{12}$ complex appears to be less than the sum of the absorption of its individual components from 260 nm to 290 nm.

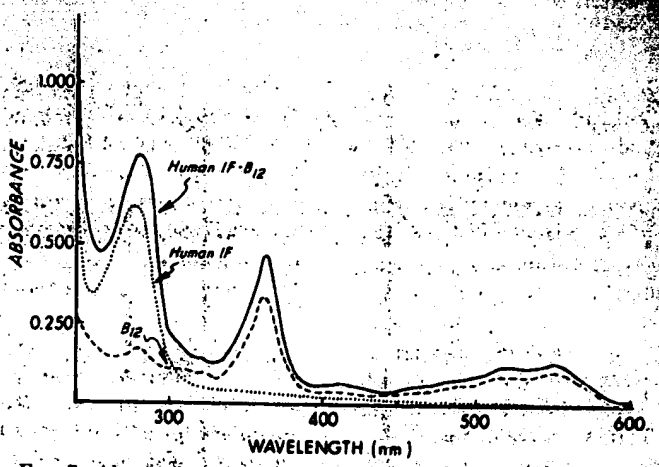


FIG. 7. Absorption spectra of human IF. ——, 546 μg per ml of human IF containing 16.4 μg per ml of vitamin $B_{12}$; ····, 546 μg per ml of human IF; – –, 16.4 μg per ml of vitamin $B_{12}$. All spectra were obtained in 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl.

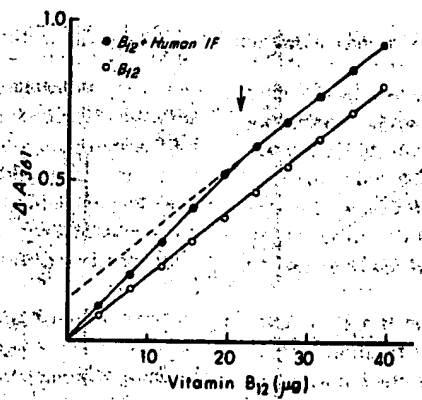


FIG. 8. Comparison of the 361 nm absorption of vitamin $B_{12}$ bound to human IF with that of unbound vitamin $B_{12}$. Aliquots of 25 μl of a solution of vitamin $B_{12}$ (153 μg per ml) in buffer (0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl) were added serially at 5-min intervals to two cuvettes. The first cuvette contained 714 μg of human IF devoid of vitamin $B_{12}$ in 0.9 ml of buffer. The second cuvette contained 0.9 ml of buffer alone. Values for $A_{361}$ were obtained for each cuvette 4 min after each addition of vitamin $B_{12}$. Values for the cumulative change in $A_{361}$ were corrected to a volume of 1.0 ml and were plotted *versus* the total amount of vitamin $B_{12}$ present. ●, values obtained for the cuvette containing 714 μg of human IF; ○, values obtained for the cuvette containing buffer alone. The *arrow* indicates the amount of vitamin $B_{12}$ required to saturate 714 μg of human IF based on its vitamin $B_{12}$-binding capacity of 30.1 μg per mg.

The amount of vitamin $B_{12}$ present in the sample of human IF-vitamin $B_{12}$ complex used to obtain the spectrum presented in Fig. 7 was determined by measuring the concentration of [$^{57}$Co]vitamin $B_{12}$. If human IF were to bind nonradioactive vitamin $B_{12}$ to a greater extent than [$^{57}$Co]vitamin $B_{12}$, it is possible that the increased absorption above 320 nm noted for the human IF-vitamin $B_{12}$ complex is an artifact. In order to evaluate a possible isotope effect, a solution of buffer containing human IF devoid of vitamin $B_{12}$ was titrated with additions of nonradioactive vitamin $B_{12}$, and the observed incremental increases in absorption at 361 nm were compared with the incremental increases observed when a solution of buffer alone was titrated with the same solution of vitamin $B_{12}$. The results of this experiment are presented in Fig. 8 and demonstrate that the increase in absorption at 361 nm is approximately 32% greater

when vitamin $B_{12}$ is added to a solution of buffer and human IF than when vitamin $B_{12}$ is added to buffer alone. The fact that this 32% greater absorption is observed only up to the point where human IF would be expected to be saturated with vitamin $B_{12}$ indicates that the 32% greater absorption is due to the binding of vitamin $B_{12}$ by human IF. The results obtained in this experiment demonstrate that the absorption of the human IF-vitamin $B_{12}$ complex at 361 nm is different from the sum of the absorption of its two components and that this difference is not due to an isotope effect.

## DISCUSSION

Affinity chromatography on vitamin $B_{12}$-Sepharose has enabled us to isolate human IF in high yield (85%) from pooled gastric juice that was collected in a manner such that contamination with other vitamin $B_{12}$-binding proteins was minimized. The final preparation of human IF appears homogeneous by sedimentation equilibrium ultracentrifugation and by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. A high degree of homogeneity is also suggested by the studies employing anti-IF antibody and pseudovitamin $B_{12}$, by the fact that a single peak of vitamin $B_{12}$-binding activity was observed on Sephadex G-150 chromatography, and finally, by the fact that 20 $\mu$g of isolated protein were able to correct vitamin $B_{12}$ malabsorption in a patient with pernicious anemia.

The ready availability of milligram amounts of homogeneous human IF has enabled us to elucidate a number of its physical properties and allows for new experiments to elucidate the mechanism by which this protein facilitates vitamin $B_{12}$ absorption in the terminal ileum. Recent experiments[2] indicate that our final preparation of human IF does facilitate vitamin $B_{12}$ binding to homogenates of guinea pig and human distal ileum. This observation, together with the fact that 20 $\mu$g of isolated protein promote vitamin $B_{12}$ absorption *in vivo*, also demonstrates that our final preparation of human IF retains its functional ability as well as its ability to bind vitamin $B_{12}$.

Gräsbeck *et al.* (9) have isolated and studied human IF as its vitamin $B_{12}$ complex. Using ion exchange chromatography, they were able to separate human IF into two fractions that were designated as Fraction S and Fraction I. Molecular weight values of 119,000 and 114,000 were obtained for Fractions S and I, respectively, using sedimentation velocity ultracentrifugation. The authors suggested that Fractions S and I both consisted of dimers of IF that contained 2 molecules of vitamin $B_{12}$ per dimer. Our observations support this suggestion since we have demonstrated that the 44,000 to 48,000 molecular weight monomeric form of human IF can form higher molecular weight oligomers in the presence of vitamin $B_{12}$. We have no definite explanation for the fact that Gräsbeck's isolated fractions appeared to consist exclusively of dimers except that human IF dimers may be very stable once formed and thus IF monomers and other higher molecular weight oligomers may have been lost during purification. This appears possible since the final recovery of IF by Gräsbeck *et al.* was in the range of 10 to 20% of the starting material.

All of our studies indicate that oligomer formation by human IF occurs only in the presence of vitamin $B_{12}$ and the studies employing polyacrylamide disc gel electrophoresis indicate that significant oligomer formation does not occur rapidly, *i.e.* not within minutes, after the addition of vitamin $B_{12}$. The fact that multiple discrete peaks of human IF-vitamin $B_{12}$ were observed during gel filtration also suggests that the monomeric and oligomeric forms of this protein are not in rapid equilibrium with each other. We have not observed complete conversion of human IF monomers to oligomers in any of our studies, and it is important to note that we have not demonstrated that this is due to a slow equilibrium between monomers and oligomers. An alternative explanation for our failure to observe complete conversion of monomers to oligomers could be that human IF is microheterogeneous, as has been noted by Gräsbeck (42), and that only certain forms are capable of forming oligomers. Oligomer formation is obviously a complex process, and additional experiments will be required to fully elucidate this phenomenon as well as to determine whether it is of any physiological significance.

Several investigators (43, 44) have determined the vitamin $B_{12}$ content of purified vitamin $B_{12}$-binding proteins by measuring the peak absorbance in the 361 nm region and then calculating the vitamin $B_{12}$ content using the 361 nm extinction coefficient for unbound vitamin $B_{12}$. Such determinations assume that the absolute absorbance of vitamin $B_{12}$ is unchanged when it is bound to protein. Our studies of human IF, human transcobalamin II (15), the human granulocyte vitamin $B_{12}$-binding protein (14), and hog gastric vitamin $B_{12}$-binding proteins (35) indicate that such an assumption would be unwarranted in every case and would result in falsely elevated values for vitamin $B_{12}$ content. The magnitude of the possible error arising from this type of assumption is illustrated by the fact that the vitamin $B_{12}$ content of our purified human IF would have been overstated by 39% if determined as described above, rather than by measuring the content of [$^{57}$Co]vitamin $B_{12}$ of known specific activity.

Immunologic studies (45, 46) using crude or partially purified protein preparations have suggested that the vitamin $B_{12}$-binding proteins found in various human body fluids can be divided into three distinct entities consisting of IF, transcobalamin II, and the R type group of proteins to which the human granulocyte vitamin $B_{12}$-binding protein belongs. We have now isolated each of the three proteins mentioned above using affinity chromatography on vitamin $B_{12}$-Sepharose, and the differences that we have observed in the properties of these proteins are consistent with their immunologic differences. Transcobalamin II (15) is the most distinct of the three proteins based on its amino acid composition, lack of any carbohydrate residues, and the fact that it alone is composed of 2 nonidentical subunits. Human IF and the human granulocyte vitamin $B_{12}$-binding protein (14) have somewhat similar amino acid compositions, contain the same type of carbohydrate residues, and appear to be composed of single polypeptide chains. These latter two proteins do differ, however, in their content of certain amino acids and carbohydrates, and this fact, together with their differences in molecular weight and spectral properties, establishes that these two proteins are also distinct entities.

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